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Novel alginates prepared by independent control of chain stiffness and distribution of G-residues: Structure and gelling properties

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ABSTRACT

The study of alginate hydrogels is of increasing interest, given their potential applications as biomaterials for tissue engineering and for encapsulating drugs and living cells. In this study, we present a new strategy for tailoring alginates on the basis of homopolymeric mannuronan, where the chain stiffness and the content of G-residues could be varied independently. Partial periodate oxidation (0–8%) followed by borohydride reduction, introducing flexible linkages through C2–C3 cleavage and ring opening, was combined with *in vitro* epimerization, introducing either alternating (MG) sequences (in the case of enzyme AlgE4) or G-blocks (in the case of enzyme AlgE6). Both enzymes are recombinantly expressed from *Azotobacter vinelandii*. Two strategies were followed: (a) oxidation/reduction followed by epimerization (b) epimerization to 90% G followed by oxidation/reduction. The resulting alginates were characterised by NMR spectroscopy and size-exclusion chromatography (SEC) with multi angular laser light scattering (MALLS) and viscosity detectors. Gels were prepared using the 'internal setting' method with either 10 mM or 20 mM Ca^{2+} present, and studied by small-strain oscillatory measurements. It was found that periodate oxidation, in the range $P_0 = 0.02–0.06$, had a pronounced influence on the gelling properties. The decrease in dynamic storage modulus (G') could mainly be attributed to increased local flexibility and not only a decrease in G-block lengths as a consequence of oxidation. The new alginate gels are easily degradable in a mild acidic environment and the degradation is easier to control than gels made of unoxidized alginate.

1. Introduction

Alginate is a family of unbranched polysaccharides consisting of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers forming regions of M-blocks (MM), G-blocks (GG) or alternating structures (MG). The guluronic acid residues are introduced *post* polymerisation by a series of C-5 epimerases. A family of seven C-5 epimerase genes, the AlgE genes, has been isolated from *Azotobacter vinelandii* and recombinantly expressed and sequenced in *Escherichia coli* (Ertesvåg, Doseth, Larsen, Skjåk-Bræk, & Valla, 1994; Ertesvåg et al., 1999; Svanem, Skjåk-Bræk, Ertesvåg, & Valla, 1999). The possibility of obtaining pure mannuronan and utilizing the C-5 epimerases *in vitro* (Mørch, Donati, Strand, & Skjåk-Bræk, 2007), in particular AlgE4 and AlgE6, is part of the background for the new alginate materials discussed in this study. AlgE4 strictly converts MM- to MG-blocks in the polymer, while AlgE6 introduces G-blocks of various lengths (Campa et al., 2004; Holtan, Bruheim, & Skjåk-Bræk, 2006). When additional G-blocks are introduced by AlgE6 they can merge, and long stretches of guluronic acid are created (Mørch, Holtan, Donati, Strand, & Skjåk-Bræk, 2008).

The structure of alginate allows the polymer chains to ionically interact with each other through divalent cations, in particular Ca^{2+}, Ba^{2+} or Sr^{2+}, hereby forming hydrogels. The complexation of alginate with divalent ions has been described by the "egg-box" model in which each divalent ion interacts with two adjacent G-residues as well as with two G-residues in an opposing chain (Grant, Morris, Rees, Smith, & Thom, 1973). Such alginate hydrogels have been intensively studied as tissue engineering scaffolds or encapsulation materials for living cells or other biologically active compounds. One of the most studied systems is the encapsulation of insulin producing cells in Ca^{2+}-alginate microcapsules for transplantation into patients to treat Type 1 diabetes (Strand, Mørch, Syvertsen, Espevik, & Skjåk-Bræk, 2003). Alginate hydrogels have also been tested in drug delivery systems. The physical properties of the gel are vital because it is important that the drug is released in a controllable manner. If it is released above or below some critical level, the drug could potentially be either toxic or inefficient (Gomez, Rinaudo, & Villar, 2007). The properties of the

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alginate hydrogels can be controlled by parameters like Ca^{2+} concentration, pH, molecular weight and the chemical composition of the alginate (Draget, Simensen, Onsøyen, & Smidsrød, 1993). In this study, we also suggest that chemical modification through periodate oxidation can be used to control gel strength.

Periodate oxidation of alginate is highly specific and results in the cleavage of the C2–C3 bond in the alginate monosaccharide units (Fig. 1A). The oxidation is assumed to act randomly upon homogenous substrates (Painter & Larsen, 1970). It is generally considered that the cleavage reaction involves reversible formation of a cyclic complex or intermediate, via an acyclic ester, and that the intermediate decomposes via a cyclic transition state to the products (Perlin, 2006). In polysaccharides the carbonyl groups formed may exist hydrated, as acyclic aldehydes, hemiacetals or hemialdals, or as combinations of these. The hemiacetal formation may be intermolecular as well as intramolecular (Gutherie, 1961; Painter & Larsen, 1970). If oxidation is followed by borohydride reduction the reactive dialdehydes are reduced to diols. Periodate oxidation of alginate also leads to some depolymerisation. The mechanism involved is presumably a free radical mediated mechanism (Balakrishnan, Lesieur, Labarre, & Jayakrishnan, 2005; Painter & Larsen, 1970), however conclusive proof of such a mechanism is still lacking. When a polysaccharide is partially oxidized by periodate the mol fraction of oxidized units compared to unoxidized units can be expressed as P_0, hence 4 mol% oxidation can be expressed as $P_0 = 0.04$.

Recently, a commercial alginate (M/G-ratio: 0.47) was periodate oxidized as a means to make alginate hydrogels with new properties (Gomez et al., 2007). It is known that the periodate oxidation of alginate has a pronounced effect upon chain extension and flexibility (Lee, Bouhadir, & Mooney, 2002; Smidsrød & Painter, 1973; Vold, Kristiansen, & Christensen, 2006), thus this should influence the gelling ability of alginate. This is confirmed in the study of Gomez et al. (2007). In this study it is concluded that the gel strength is rapidly decreasing with increasing P_0 and reaching $P_0 = 0.10$ no gelling takes place in an excess of calcium. However, in the experiment of Gomez et al. (2007) some of the oxidation sites were within G-blocks and these consequently split the G-block. The G-block length ($N_{G>1}$) is important in controlling gel strength (Draget et al., 1993). Performing periodate oxidation therefore introduces both increased local flexibility and a reduction in G-block length, thus it is not concluded whether the increased flexibility alone affects the gel strength. It is indeed pointed out that the molecular weight is rapidly decreasing during periodate oxidation, also being an important factor in gel formation (Draget et al., 1993; Draget et al., 2000).

The degradation of partially oxidized alginate and its potential application for tissue engineering has also been investigated (Bouhadir et al., 2001). By partially oxidizing alginate the hydrogels formed can be degraded in a more controlled fashion, since the oxidized residues are much more susceptible to degradation than the unoxidized residues (Bouhadir et al., 2001). The increased susceptibility to acid hydrolysis of periodate oxidized and borohydride reduced oligo- and polysaccharides is well known and often applied in Smith degradation (Abdelakher, Hamilton, Montgomery, & Smith, 1952). Periodate oxidized polysaccharides in general are also more susceptible to alkaline degradation, demonstrated with both periodate oxidized dermatan sulphate and cellulose (Calvini, Conio, Lorenzoni, & Pedemonte, 2004; Fransson & Carlstedt, 1974).

In this study, we first investigated the structural outcome of periodate oxidized mannuronan. Periodate oxidized and subsequently borohydride reduced mannuronan was further used as a substrate for the C-5 epimerases AlgE4 and AlgE6 (Fig. 1A). The gel strength and gelling kinetics of hydrogels formed of mannuronan epimerized with AlgE6 after or before periodate oxidation ($P_0 = 0.02–0.08$) and reduction were studied performing small-strain oscillatory measurements (Fig. 1B). The mannuronan epimerized after oxidation results in a new alginate material with long G-blocks that were interspersed by oxidized mannuronan residues possessing increased local flexibility. Assuming random oxidation the G-block length was dictated by the number of mannuronan residues between the oxidized units, but limited by the minimum number of residues needed for the enzyme to attack. It was assumed that the enzyme was specific and did not attack oxidized residues. Mannuronan epimerized prior to oxidation had extremely long G-blocks interspersed by oxidized units. These materials were compared with alginate from *Laminaria hyperborea* and *Durvillea antarctica* having comparable G-block lengths which made it possible to study whether it was the increased flexibility introduced by the periodate oxidation or a reduction in G-block length accompanying the oxidation that caused the drop in the dynamic storage modulus (G'). Finally, it was investigated whether periodate oxidation could be used to 'tune' gel strength, potentially resulting in new easily degradable alginate gels.

2. Materials and methods

2.1. Materials and chemicals

Mannuronan and alginates from *D. antarctica* and *L. hyperborea* were obtained from FMC Biopolymer, Drammen, Norway. The mannuronan was further purified by precipitation in 50% ethanol, filtration and two cycles of washing with 98% ethanol. The other alginates used in this study were obtained by epimerizing mannuronan with the C-5 epimerases AlgE4 and AlgE6, obtained as described earlier (Campa et al., 2004; Holtan et al., 2006). All chemicals were obtained from commercial sources and were of analytical grade.

2.2. Periodate oxidation and borohydride reduction of mannuronan/alginate

Mannuronan/alginate was dissolved in MQ-water (deionized water purified with the MilliQ system from Millipore (Bedford, MA, USA)) to a concentration of 8.89 mg/mL. The solution was then made up with 10% (v/v) *n*-propanol (free radical scavenger) and MQ-water. Degassing (nitrogen) was preformed prior to the addition of 0.25 M sodium meta periodate in order to obtain mannuronan/alginate with P_0 (periodate/monomer ratio) of 0.02–0.08. The final polysaccharide concentration was 4.45 mg/mL. All pipetting and weighing were performed in subdued light and the reaction was carried out at 20 °C.

After periodate oxidation, sodium borohydride was added to a final concentration of 20% (w/v) and left for 2 h at RT. The samples were then placed on ice and concentrated acetic acid was added until all hydrogen gas had effervesced. The samples were further adjusted to pH 7 and dialysed against MQ-water at 4 °C until the conductivity was below 4 μS. Finally the samples were freeze–dried.

2.3. Epimerization

In the first part of the study periodate oxidized mannuronan ($P_0 = 0.05–0.20$) was used as the substrate for the epimerases AlgE4 and AlgE6. The substrate was dissolved in MQ-water overnight before a concentrated stock solution of MOPS buffer (pH 6.9) with $CaCl_2$ monohydrate and NaCl were added, and the mixtures were pre-heated at 37 °C. The respective enzymes were dissolved in MQ-water and immediately added to the substrate solutions. Final concentrations of the reaction mixtures were 0.25% (w/v) substrate, 50 mM MOPS, 2.5 mM $CaCl_2$ and 10 mM NaCl. The epimerization reaction was left for 20 h and stopped by lowering the pH to 3 with cold HCl.